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The present invention concerns a novel epitope displayed by the N–terminus of the precursor of brain natriuretic peptide (NT–proBNP), its use in diagnostic tests for left ventricular dysfunction, diagnostic test methods and kits for same.

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NATRIURETIC PEPTIDE FRAGMENTS

The present invention concerns a novel epitope displayed by the N-terminus of the precursor of brain natriuretic peptide (NT-proBNP), its use in diagnostic tests for left ventricular dysfunction, diagnostic test methods and kits for same.

Mukoyama and co-workers published work in 1991 which suggested that brain natriuretic peptide (BNP) and another hormone atrial natriuretic peptide (ANP) may be correlated with heart failure or incipient heart failure (Mukoyama, M. *et al.*, 1991a, J. Clin. Invest., 87(4): 1402-1412; Mukoyama, M. *et al.*, 1991b, Biochem. Biophys. Res. Commun., 180(1): 431-436). WO93/24531 discloses antibody to the 76 residue N-terminal end of BNP (NT-proBNP), for use in diagnosing or prognosing heart failure or hypervolemia. More recent research substantiated these earlier suggestions, confirming the role of BNP, together with other hormones such as ANP and N-terminal proANP (NT-proANP), as indices of left ventricular dysfunction (LVD) and as prognostic indicators of outcome following acute myocardial infarction (Omland, T. *et al.*, 1996, Circulation, 93: 1963-1969; McDonagh, T. A. *et al.*, 1998, The Lancet, 351: 9-13

Richards, A. M. *et al.* (1998, Circulation, 97 (19): 1921-1929) state that NT-proBNP (also referred to as N-BNP) is a good indicator of left ventricular function both early and late after myocardial infarction, and of cardiovascular prognosis.

Human BNP-32 is the natural form of the 108 amino acid proBNP precursor (pre-proBNP), correlating to amino acids 77-108 of pre-proBNP. NT-proBNP correlates to amino acids 1-76 of pre-proBNP. Hunt, P. J. *et al.* (1997, Clinical Endocrinology, 47: 287-296) confirms both BNP and NT-proBNP as being indicators of cardiac impairment,

and of NT-proBNP as being a more discerning marker of early cardiac dysfunction than BNP. WO 97/32900 discloses antibody specific to epitopes comprising amino acids 1-10, 5-13 and 15-25 of human BNP.

The present inventor has succeeded in identifying a previously unknown epitope located away from the previously identified epitopes on NT-proBNP.

The present inventor has identified the epitope having the amino acid sequence of SEQ ID NO: 1 and found it to be particularly diagnostically useful. Thus according to a first aspect of the present invention there is provided a peptide identical to an epitope having the amino acid sequence of SEQ ID NO: 1. Also provided is the epitope having the amino acid sequence of SEQ ID NO: 1, displayed by NT-proBNP. The peptide may have the amino acid sequence of SEQ ID NO: 1 or it may for example be a mimotope (Geysen, H. M. *et al.*, 1988, J. Mol. Recognit., 1 (1): 32-41) of such an epitope.

SEQ ID NO: 1 represents amino acids 65-76 of the NT-proBNP sequence, located at the carboxy terminus of the NT-proBNP sequence.

As disclosed in WO 97/32900 (*supra*), epitopes have to date been found at the N-terminus of the BNP molecule rather than the C-terminal of NT-proBNP, and there has been no suggestion of their being located at the C-terminus.

The epitope (peptide) of the present invention may be for use in a method of diagnosis of NT-proBNP. Also provided is the use of a peptide of the present invention in the manufacture of a diagnostic test for NT-proBNP. Also provided is a method of manufacture of a diagnostic test for NT-proBNP, characterised in the use of a peptide according to the present invention. The diagnostic test for NT-proBNP using

a peptide according to the present invention may be performed within the range of about 70 to 120 hours following acute myocardial infarction. Such diagnostic tests and methods may include e.g. competitive binding assays.

Since NT-proBNP levels are indicative of left ventricular dysfunction, diagnostics may be tests for left ventricular dysfunction.

Also provided is a diagnostic test method for left ventricular dysfunction, comprising the steps of:

i) determining the quantity of an epitope according to the present invention in a sample from a patient;

ii) comparing the level of epitope determined in step (i) with the level of epitope determined in another patient having a known left ventricular function; and

iii) correlating the results of comparison step (ii) with left ventricular dysfunction of the patient.

Such diagnostic tests typically use antibodies or antigen binding fragments (Harlow, E. and Lane, D., 1988, "Antibodies - A Laboratory Press, New York) to achieve the detection of the epitope of the present invention and the determination of its quantity in a sample, and reference herein to antibody or antibodies is also reference to antigen binding fragments thereof.

Thus the present invention also provides antibodies specific against a peptide according to the present invention (the epitope of the present invention displayed

by NT-proBNP is the same as that displayed by the peptide). Also provided is the use of such an antibody in the manufacture of a diagnostic test for NT-proBNP, e.g. for left ventricular dysfunction. Also provided is a method of manufacture of a diagnostic test for NT-proBNP, e.g. for left ventricular dysfunction characterised in the use of an antibody according to the present invention. The diagnostic test employing antibody according to the present invention may be performed within the range of about 70 to 120 hours following acute myocardial infarction.

The conditions in which such an assay would be useful include:-

1) post acute myocardial infarction, to detect systolic dysfunction which is correlated with mortality
2) detection of left ventricular systolic dysfunction in the diagnosis and monitoring of congestive cardiac failure

3) detection of diastolic dysfunction including valvular regurgitation

Thus, the present invention also provides a diagnostic test method for NT-proBNP. comprising the steps of:

i) reacting antibody specific to the epitope of the present invention with a sample from a patient;

ii) detecting an antibody - antigen binding reaction; and

iii) correlating detection of the antibody-antigen binding reaction with the presence of NT-proBNP.

The diagnostic test method may be used to simply determine whether or not NT-proBNP is present in a sample, or it may be used to determine the level (i.e. quantity) of NT-proBNP present in a sample.

Such a diagnostic test method for NT-proBNP could be used to determine the level of epitope in steps (i) and/or (ii) of the above diagnostic test method for left ventricular dysfunction.

To date, all assays for BNP and NT-proBNP have used radio labelled ligands in a competitive binding assay format. Such ligands are often unstable and have to be utilised before radioactive decay renders them useless. Moreover, radiation protection and disposal add to the cost and inconvenience of such assays.

The location of the epitope of the present invention at the carboxy terminus of NT-proBNP allows for the development of an effective two-site assay for NT-proBNP. The two-site assay format is particularly useful in that it allows the determination of NT-proBNP levels in unextracted plasma, the epitope of the present invention not being displayed by pre-proBNP. By "unextracted plasma" is meant that there is no need to use an extraction column to concentrate a plasma sample prior to performing the assay.

As a second site, the epitope displayed by amino acids 1-13 (SEQ ID NO: 2) of the NT-proBNP sequence is particularly useful.

A particularly useful feature of the two site assay format is that it need not involve the use of radio labelled reagents. For example chemiluminescent or immunoluminescent reagents may be used instead, typically being much more convenient and less costly. One particular chemiluminescent label which may be used

to label antibodies is 4-(2-succinimidyloxycarbonyl ethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulfonate. This chemiluminescent label has previously been described for the development of competitive and two-site assays (Weeks, I. *et al.*, 1983, Clin. Chem., 29: 1474-1479; Weeks, I. *et al.*, 1986, Methods Enzymol., 133: 366-387), its use in labelling small peptides such as NT-proBNP has not previously been described.

It is believed that the use of the epitope of the present invention, which is distant from previously identified epitopes, allows for an efficient and effective two-site assay, which may not have been possible to achieve using antibodies against a pair of prior art epitopes due to the proximity of such epitopes and the steric hindrance which the binding of a first antibody would cause upon the binding of a second antibody. In addition, the levels of NT-proBNP rise more than those of BNP-32 and are therefore more easily detectable in plasma than BNP-32.

Thus also provided according to the present invention is the use of antibody specific against the epitope of the present invention, together with antibody specific against a different epitope displayed by NT-proBNP in the manufacture of a two-site diagnostic test for NT-proBNP.

Also provided is a diagnostic test method for NT-proBNP comprising the steps of:

i) reacting with a sample from a patient a first antibody specific to either one of the group of the epitope of the present invention or a different epitope displayed by NT-proBNP;

ii) reacting with sample bound to antibody in step (i) a second antibody specific to the other of the group of the epitope of the present invention and a different epitope displayed by NT-proBNP;

iii) detecting binding of the antibody of step (ii) with antigen; and

iv) correlating the results of detection step (iii) with the presence of NT-proBNP.

The second antibody may be labelled with a chemiluminescent marker or another chosen marker, for example a non-radioactive marker.

Naturally, agents other than antibodies may be used as long as they are capable of specifically binding to epitopes of NT-proBNP as required.

The following recent papers relevant to the present invention and co-authored by the present inventor are incorporated herein by reference in their entirety: (i) Hughes *et al.* (1999a) Clin. Sci. (Colch.) 96(4): 373-380; (ii) Downie, P.F. *et al.* (1999b) Clin. Sci. (Colch.) 97(3): 255-258; and (iii) Talwar, S. *et al.* (1999c) Eur. Heart J. 20(23): 1736-1744.

The invention will be further apparent from the following description, with reference to the several figures of the accompanying drawings, which show, by way of example only, forms of assay for NT-proBNP.

Of the Figures:

Figure 1 shows the relationship of the chemiluminescence of immunoprecipitates using different concentrations of antibodies G172 (for peptide NT1) and G185 (for peptide NT2) in the immunoluminometric assay. Approximately 10^6 RLU of the labelled peptide NT1 or NT2 were added per tube at the beginning of the assay. X-axis shows the amount of antibody added (ng) and Y-axis shows relative light units (RLU);

Figure 2 shows competitive binding curves for different added amounts of the peptides NT1 and NT2 reacted with 20 ng per tube of the specific antibodies G172 and G185. The chemiluminescence values were corrected to a value of 1 in the tubes with no added peptide. X-axis shows amount of peptide added (fmol) and Y-axis shows B/Bo;

Figure 3 shows competitive binding curve for the peptide NT2, with 20 ng of the antibody G185 added per tube. Examples of 2 plasma samples from cardiac failure patents and 1 from a normal control taken through a series of dilutions are also plotted as hollow symbols with dotted lines. X-axis shows NT2 (fmol) and Y-axis shows B/Bo;

Figure 4 shows plasma levels of log NT-proBNP (measured using an antibody against peptide NT2) plotted against the left ventricular wall motion index (LVWMI) for patients on an ACE inhibitor and/or a diuretic (Top graph) and patients who were not on an ACE inhibitor or a diuretic (Lower graph);

Figure 5 shows a box plot of the relationship between log NT-proBNP levels (measured using an antibody against peptide NT2) and the severity of mitral regurgitation.

Figure 6 shows a profile of plasma NT-proBNP (measured using an antibody against peptide NT2) following anterior (■) or inferior (o) myocardial infarction;

Figure 7 shows the correlation of Wall Motion Index during hospitalisation and NT-proBNP (measured using an antibody against peptide NT2), sampled at 73-120 hours following myocardial infarction; and

Figure 8 shows individual plasms NT-proBNP (measured using an antibody against peptide NT2) in patients with poor outcome (WMI ≤ 1.2 or death) (□) and others (o) following myocardial infarction. Horizontal bars indicate group mean value.

Experimental

EXAMPLE 1: Isolation and comparative testing of antibodies to NT-proBNP epitopes

METHODS

Materials

Peptides corresponding to amino acids 37-49 (SEQ ID NO: 3) and amino acids 65-76 (SEQ ID NO: 1) of the preproBNP sequence, representing the mid section and C-terminal respectively of NT-proBNP were synthesised. These are referred to hence as NT1 and NT2 respectively. Protein A Sepharose C14B gel was obtained from Pharmacia, Hertfordshire. The methyl acridinium ester (4-(2-succinimidyloxycarbonyl ethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulfonate) was from Molecular Light Technology Ltd., Cardiff. The paramagnetic particles coated with goat anti-rabbit IgG were from Metachem Diagnostics Ltd., Northampton. The C_{18} plasma extraction columns and the peptides atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and proBNP(22-46) were obtained from Peninsula laboratories, Merseyside. All other reagents were of Analar grade and obtained from Sigma Chemical Co. Ltd., Poole.

Production of Antibodies

The NT1 and NT2 peptides were conjugated to haemocyanin with the heterobifunctional cross linker ε-maleimidocaproic acid N-hydroxysuccinimde ester. Two rabbits inoculated with subcutaneous injections of antigens emulsified with complete Freund's adjuvant. After a month, booster injections were given intravenously every 2 weeks and the antisera obtained after 3 months. The IgG fraction was obtained by protein A

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The present inventor has succeeded in identifying a previously unknown epitope located away from the previously identified epitopes on NT-proBNP.

The present inventor has identified the epitope having the amino acid sequence of SEQ ID NO: 1 and found it to be particularly diagnostically useful. Thus according to a first aspect of the present invention there is provided a peptide identical to an epitope having the amino acid sequence of SEQ ID NO: 1. Also provided is the epitope having the amino acid sequence of SEQ ID NO: 1, displayed by NT-proBNP. The peptide may have the amino acid sequence of SEQ ID NO: 1 or it may for example be a mimotope (Geysen, H. M. *et al.*, 1988, J. Mol. Recognit., 1 (1): 32-41) of such an epitope.

SEQ ID NO: 1 represents amino acids 65-76 of the NT-proBNP sequence, located at the carboxy terminus of the NT-proBNP sequence.

As disclosed in WO 97/32900 (*supra*), epitopes have to date been found at the N-terminus of the BNP molecule rather than the C-terminal of NT-proBNP, and there has been no suggestion of their being located at the C-terminus.

The epitope (peptide) of the present invention may be for use in a method of diagnosis of NT-proBNP. Also provided is the use of a peptide of the present invention in the manufacture of a diagnostic test for NT-proBNP. Also provided is a method of manufacture of a diagnostic test for NT-proBNP, characterised in the use of a peptide according to the present invention. The diagnostic test for NT-proBNP using

a peptide according to the present invention may be performed within the range of about 70 to 120 hours following acute myocardial infarction. Such diagnostic tests and methods may include e.g. competitive binding assays.

Since NT-proBNP levels are indicative of left ventricular dysfunction, diagnostics may be tests for left ventricular dysfunction.

Also provided is a diagnostic test method for left ventricular dysfunction, comprising the steps of:

i) determining the quantity of an epitope according to the present invention in a sample from a patient;

ii) comparing the level of epitope determined in step (i) with the level of epitope determined in another patient having a known left ventricular function; and

iii) correlating the results of comparison step (ii) with left ventricular dysfunction of the patient.

Such diagnostic tests typically use antibodies or antigen binding fragments (Harlow, E. and Lane, D., 1988, "Antibodies - A Laboratory Press, New York) to achieve the detection of the epitope of the present invention and the determination of its quantity in a sample, and reference herein to antibody or antibodies is also reference to antigen binding fragments thereof.

Thus the present invention also provides antibodies specific against a peptide according to the present invention (the epitope of the present invention displayed

by NT-proBNP is the same as that displayed by the peptide). Also provided is the use of such an antibody in the manufacture of a diagnostic test for NT-proBNP, e.g. for left ventricular dysfunction. Also provided is a method of manufacture of a diagnostic test for NT-proBNP, e.g. for left ventricular dysfunction characterised in the use of an antibody according to the present invention. The diagnostic test employing antibody according to the present invention may be performed within the range of about 70 to 120 hours following acute myocardial infarction.

The conditions in which such an assay would be useful include:-

1) post acute myocardial infarction, to detect systolic dysfunction which is correlated with mortality
2) detection of left ventricular systolic dysfunction in the diagnosis and monitoring of congestive cardiac failure

3) detection of diastolic dysfunction including valvular regurgitation

Thus, the present invention also provides a diagnostic test method for NT-proBNP. comprising the steps of:

i) reacting antibody specific to the epitope of the present invention with a sample from a patient;

ii) detecting an antibody - antigen binding reaction; and

iii) correlating detection of the antibody-antigen binding reaction with the presence of NT-proBNP.

The diagnostic test method may be used to simply determine whether or not NT-proBNP is present in a sample, or it may be used to determine the level (i.e. quantity) of NT-proBNP present in a sample.

Such a diagnostic test method for NT-proBNP could be used to determine the level of epitope in steps (i) and/or (ii) of the above diagnostic test method for left ventricular dysfunction.

To date, all assays for BNP and NT-proBNP have used radio labelled ligands in a competitive binding assay format. Such ligands are often unstable and have to be utilised before radioactive decay renders them useless. Moreover, radiation protection and disposal add to the cost and inconvenience of such assays.

The location of the epitope of the present invention at the carboxy terminus of NT-proBNP allows for the development of an effective two-site assay for NT-proBNP. The two-site assay format is particularly useful in that it allows the determination of NT-proBNP levels in unextracted plasma, the epitope of the present invention not being displayed by pre-proBNP. By "unextracted plasma" is meant that there is no need to use an extraction column to concentrate a plasma sample prior to performing the assay.

As a second site, the epitope displayed by amino acids 1-13 (SEQ ID NO: 2) of the NT-proBNP sequence is particularly useful.

A particularly useful feature of the two site assay format is that it need not involve the use of radio labelled reagents. For example chemiluminescent or immunoluminescent reagents may be used instead, typically being much more convenient and less costly. One particular chemiluminescent label which may be used

to label antibodies is 4-(2-succinimidyloxycarbonyl ethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulfonate. This chemiluminescent label has previously been described for the development of competitive and two-site assays (Weeks, I. *et al.*, 1983, Clin. Chem., 29: 1474-1479; Weeks, I. *et al.*, 1986, Methods Enzymol., 133: 366-387), its use in labelling small peptides such as NT-proBNP has not previously been described.

It is believed that the use of the epitope of the present invention, which is distant from previously identified epitopes, allows for an efficient and effective two-site assay, which may not have been possible to achieve using antibodies against a pair of prior art epitopes due to the proximity of such epitopes and the steric hindrance which the binding of a first antibody would cause upon the binding of a second antibody. In addition, the levels of NT-proBNP rise more than those of BNP-32 and are therefore more easily detectable in plasma than BNP-32.

Thus also provided according to the present invention is the use of antibody specific against the epitope of the present invention, together with antibody specific against a different epitope displayed by NT-proBNP in the manufacture of a two-site diagnostic test for NT-proBNP.

Also provided is a diagnostic test method for NT-proBNP comprising the steps of:

i) reacting with a sample from a patient a first antibody specific to either one of the group of the epitope of the present invention or a different epitope displayed by NT-proBNP;

ii) reacting with sample bound to antibody in step (i) a second antibody specific to the other of the group of the epitope of the present invention and a different epitope displayed by NT-proBNP;

iii) detecting binding of the antibody of step (ii) with antigen; and

iv) correlating the results of detection step (iii) with the presence of NT-proBNP.

The second antibody may be labelled with a chemiluminescent marker or another chosen marker, for example a non-radioactive marker.

Naturally, agents other than antibodies may be used as long as they are capable of specifically binding to epitopes of NT-proBNP as required.

The following recent papers relevant to the present invention and co-authored by the present inventor are incorporated herein by reference in their entirety: (i) Hughes *et al.* (1999a) Clin. Sci. (Colch.) 96(4): 373-380; (ii) Downie, P.F. *et al.* (1999b) Clin. Sci. (Colch.) 97(3): 255-258; and (iii) Talwar, S. *et al.* (1999c) Eur. Heart J. 20(23): 1736-1744.

The invention will be further apparent from the following description, with reference to the several figures of the accompanying drawings, which show, by way of example only, forms of assay for NT-proBNP.

Of the Figures:

Figure 1 shows the relationship of the chemiluminescence of immunoprecipitates using different concentrations of antibodies G172 (for peptide NT1) and G185 (for peptide NT2) in the immunoluminometric assay. Approximately 10^6 RLU of the labelled peptide NT1 or NT2 were added per tube at the beginning of the assay. X-axis shows the amount of antibody added (ng) and Y-axis shows relative light units (RLU);

Figure 2 shows competitive binding curves for different added amounts of the peptides NT1 and NT2 reacted with 20 ng per tube of the specific antibodies G172 and G185. The chemiluminescence values were corrected to a value of 1 in the tubes with no added peptide. X-axis shows amount of peptide added (fmol) and Y-axis shows B/Bo;

Figure 3 shows competitive binding curve for the peptide NT2, with 20 ng of the antibody G185 added per tube. Examples of 2 plasma samples from cardiac failure patents and 1 from a normal control taken through a series of dilutions are also plotted as hollow symbols with dotted lines. X-axis shows NT2 (fmol) and Y-axis shows B/Bo;

Figure 4 shows plasma levels of log NT-proBNP (measured using an antibody against peptide NT2) plotted against the left ventricular wall motion index (LVWMI) for patients on an ACE inhibitor and/or a diuretic (Top graph) and patients who were not on an ACE inhibitor or a diuretic (Lower graph);

Figure 5 shows a box plot of the relationship between log NT-proBNP levels (measured using an antibody against peptide NT2) and the severity of mitral regurgitation.

Figure 6 shows a profile of plasma NT-proBNP (measured using an antibody against peptide NT2) following anterior (■) or inferior (○) myocardial infarction;

Figure 7 shows the correlation of Wall Motion Index during hospitalisation and NT-proBNP (measured using an antibody against peptide NT2), sampled at 73-120 hours following myocardial infarction; and

Figure 8 shows individual plasms NT-proBNP (measured using an antibody against peptide NT2) in patients with poor outcome (WMI ≤ 1.2 or death) (□) and others (○) following myocardial infarction. Horizontal bars indicate group mean value.

Experimental

EXAMPLE 1: Isolation and comparative testing of antibodies to NT-proBNP epitopes

METHODS

Materials

Peptides corresponding to amino acids 37-49 (SEQ ID NO: 3) and amino acids 65-76 (SEQ ID NO: 1) of the preproBNP sequence, representing the mid section and C-terminal respectively of NT-proBNP were synthesised. These are referred to hence as NT1 and NT2 respectively. Protein A Sepharose C14B gel was obtained from Pharmacia, Hertfordshire. The methyl acridinium ester (4-(2-succinimidyloxycarbonyl ethyl)phenyl-10-methylacridinium 9-carboxylate fluorosulfonate) was from Molecular Light Technology Ltd., Cardiff. The paramagnetic particles coated with goat anti-rabbit IgG were from Metachem Diagnostics Ltd., Northampton. The C_{18} plasma extraction columns and the peptides atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and proBNP(22-46) were obtained from Peninsula laboratories, Merseyside. All other reagents were of Analar gradc and obtained from Sigma Chemical Co. Ltd., Poole.

Production of Antibodies

The NT1 and NT2 peptides were conjugated to haemocyanin with the heterobifunctional cross linker ε-maleimidocaproic acid N-hydroxysuccinimde ester. Two rabbits inoculated with subcutaneous injections of antigens emulsified with complete Freund's adjuvant. After a month, booster injections were given intravenously every 2 weeks and the antisera obtained after 3 months. The IgG fraction was obtained by protein A sepharose chromatography. Antibody G172 reacted to peptide NT1 and G185 with the peptide NT2.

Peptide Labelling with the methyl acridinium ester

The peptides NT1 and NT2 were dissolved in 100 mmol/l Na_2HPO_4 buffer, pH 8 at a concentration of 200 µmol/l and 100µl pipetted into an Eppendorf tube. 5 µg of the methyl acridinium ester was dissolved in 5 µl of dimethylformamide and mixed with the peptide to be labelled. After incubation at room temperature for 30 minutes in the dark, 100 µl of a lysine quench solution (10 mg/ml in 100 mmol/l Na_2HPO_4 buffer pH 8) was added and incubated for another 5 minutes. The labelled peptide solution was acidified with an equal volume of 1% trifluoroacetic acid (TFA). An aliqout of this was then injected into a 3.9mm x 150mm Deltapak C_{18} 300 Angstrom column, mounted within a high performance liquid chromatography (HPLC) system (Waters, Watford, Hertfordshire) consisting of a Waters 600S controller, 626 pump and 486 tunable absorbance detector. The column was equilibrated with 0.1% TFA and a gradient of acetonitrile from 0 to 55% (at a rate of 2%/minute) was used to elute peptides. NT1 and NT2 were eluted at 35 and 35.5% acetonitrile respectively. Their respective methyl acridinium esters were eluted at 45 and 47% acetonitrile. The hydrophobicity of the labelled peptides following derivatisation with the methyl acridinium ester facilitated the separation from unlabelled peptide. These fractions were collected and used for development of the immunoluminometric assays (ILMA). Fractions collected at other times (corresponding to $OD_{205\,nm}$ absorbance peaks) were inactive. The labelled peptides were stored in the dark at -70°C in the 0.1%TFA buffer, being stable for over 6 months.

Subjects

Blood was obtained from normal controls or patients with LVD. Normal controls were out-patients who were scanned for suspected murmurs but were found to have no significant cardiovascular abnormality. Wall motion index (WMI) using a nine segment echocardiographic model (see below) was used to assess left ventricular function. LVD was defined as a WMI of less than 1.4

20 ml of blood was transferred to chilled tubes containing 500 IU/ml aprotinin (Trasylol, Bayer UK, Newbury) and EDTA (1.5 mg/ml). Following centrifugation, plasma was stored at -70°C until the assay was performed. All samples were analysed within 2 months of venesection.

Plasma specimens were defrosted and acidified with an equal volume of 1% TFA. After centrifugation, the supernatant was loaded onto C_{18} extraction columns. Following washes with 0.1% TFA, the peptides were eluted with 0.1% TFA containing 60% acetonitrile. The eluates were dried in a centrifugal evaporator.

Immunoluminometric assay for NT-proBNP

The ILMA assay buffer consisted of (in mmol/l) NaH_2PO_4 1.5, Na_2HPO_4 8, NaCl 140, EDTA 1 and (in g/l) bovine serum albumin 1, azide 0.1. Wash buffer was composed of (in mmol/l) NaH_2PO_4 1.5, Na_2HPO_4 8, NaCl 140, and (in g/l) Tween 20 0.5, gelatin 1, azide 0.1 .

On day 1 of the assay, 100 µl of assay buffer containing 20 ng of the antibodies G172 or 185 were pipetted into tubes and incubated overnight at 4°C with peptide standards in the range 1 - 2000 fmol per tube. 100 µl of assay buffer containing about 10^6 relative light units (RLU) of the labelled peptide NT1 or NT2 was then added and tubes again incubated overnight at 4°C. On day 3, paramagnetic particles coated with goat anti-rabbit IgG was added to tubes to recover the immunoprecipitates. The particles with attached immunoprecipitates were washed 3 times in a magnetised tube rack, allowing the wash solutions to drain adequately. Reading of the chemiluminescence from the immunoprecipitates were then obtained on a Lumino portable luminometer (Stratec Electronic GMBH, Birkenfeld, Germany). In order to initiate chemiluminescence of the label the first injection was 100 µl of 100 mmol/l HNO_3 containing 0.05% hydrogen peroxide, followed 4 seconds later by an injection of 100 µl of 250 mmol/l NaOH

containing 0.25% cetyl triethylammonium bromide. The detergent optimised the light emission from the label. Chemiluminescence was measured over 2 seconds following the second injection and expressed as RLU. Standard curves were obtained and non-linear least squares fitting performed using a least-squares algorithm (P-Fit, Biosoft, Cambridge) with a Rodbard 4 parameter equation.

Echocardiography

Echocardiography was performed using a Hewlett Packard Sonos 1500 imaging system and recordings were on Panasonic Super VHS tapes. Left ventricular wall motion index (LVWMI, a regional measurement of LVD) which has been shown to be closely correlated to LVEF by radionuclide cardiography and invasive ventriculography was calculated using a nine-segment model which was originally described by Heger, J. J. *et al.* (1980, Circulation, 61: 1113-1118). The scale used for the LVWMI has been validated and a linear correlation between LVWMI and LVEF (Berning, J. *et al.*, 1992 Cardiology, 80: 257-266), has been previously demonstrated Kober, L. *et al.*, (1994, Eur, Heart J., 15: 1616-1620). LVWMI multiplied by 0.3 gives an estimate of LVEF. This nine segment model for calculation of LVWMI was used in the large multicentre TRACE study (Kober, L. *et al.*, 1995, N. Engl. J. Med., 333: 1670-1676).

The scanning protocol consisted of obtaining the following views :- (A) Parasternal long axis sector of left ventricle, (B) Parasternal short axis at mitral valve level, (C) Parasternal short axis at papillary muscle level, (D) Apical 4 chamber view, (E) Apical long axis view. LVMMI was analysed blind to the patient details.

Statistics

All results are expressed as medians [ranges] and comparisons were by the Mann Whitney test, performed on an Oxstat statistics package (Microsoft Corporation, Reading, U.K.). Two tailed P values under 0.05 were considered significant.

RESULTS

The peptides NT1 and NT2 were labelled to high specific activity using the methyl acridinium ester and HPLC was essential to separate labelled from unreacted peptide. Peaks recovered at other acetonitrile concentrations on the gradient were unreactive with the antibodies. Although 10^6 RLU of label were added per tube, the non-specific binding was under 1000 RLU using the paramagnetic particles to recover immunoprecipitates and with the washing protocol described above. The quantity of antibody added to tubes was titrated so that a non-saturating amount was added to each tube. Figure 1 illustrates that RLU measured in immunoprecipitates recovered with differing amounts of G172 or G185. On this basis, 20 ng was chosen as a suitable antibody amount.

Standard curves were constructed using known amounts of NT1 and NT2 and 20 ng of the antibodies G172 and G185. 10^6 RLU of label was subsequently added. Chemiluminescence of the recovered immunoprecipitates with differing amounts of added peptide standard is plotted in Figure 2. Increasing concentrations of each peptide displaced the label from the immunoprecipitates. These displacement curves were unchanged by the addition of 1 mol/l NaCl to the ILMA buffer or by changing the pH to 5.0 (data not shown).

When pooled plasma from 6 normal controls was compared to that of 6 patients with NYHA class III or IV congestive cardiac failure, the mean level of plasma NT1 (measured with G172) was 12 fmol/ml compared to 18.8 fmol/ml respectively. These same pooled specimens were assayed for NT2 (with G185) and a level of 170 fmol/ml was recorded in normal controls as compared to 1311 fmol/ml in the congestive cardiac failure pooled plasma. Studies on NT1 in the extracts from pooled plasma in the presence of 0.1% sodium dodecyl sulphate (to denature the peptide) revealed no differences between the normal controls and the congestive cardiac failure patients.

Further evaluation of the assay for NT2 indicated little cross reactivity with other peptides. Cross reactivities were <0.1% for ANP, BNP, CNP or proBNP(22-46). Within assay and between assay coefficients of variation for different concentrations of NT2 are reported in Table 1.

Table 1. Assay coefficients of variation for ILMA of NT2.

Within Assay Coefficients of Variation

fmol NT2	Coefficient of Variation %
12.5	3.4
25	3.0
50	3.6
100	6.6
200	6.0

Between Assay Coefficients of Variation

fmol NT2	Coefficient of Variation %
3.3	5.8
10	5.3
30	11.2
90	8.9
270	20.2
810	18.5
2430	18.8

Figure 3 illustrates a competitive binding curve for NT2 together with 2 plasma extracts from patients with congestive cardiac failure, diluted with ILMA assay buffer. The

dilution curves are almost parallel within the working range of the assay (between about 5 and 200 fmol/tube).

NT2 levels were compared in plasma from 12 patients with LVD (median age 72 [56-87] years, 5 males, median LVWMI 0.9 [0.3-1.4]) and 12 patients with no cardiovascular history (38 [20-79] years, 5 male, LVWMI 2.0 for all). Plasma NT2 levels were elevated (639 [386-911] fmol/ml) in patients with LVD as compared to normal controls (159 [120-245] fmol/ml) ($P<0.001$ by Mann Whitney test).

DISCUSSION

There is much current interest in plasma BNP as a prognostic marker for cardiovascular events and as a non-invasive diagnostic test for LVD. By analogy with NT-ANP in the ANP system, NT-proBNP has been postulated to rise to a greater extent than BNP-32 in LVD. Recently, NT-proBNP has been described to be elevated after AMI and predicts 2 year survival. Advantages of measuring NT-proBNP include the greater amounts present in the plasma of patients with congestive cardiac failure, potentially facilitating more accurate measurement. A non-radioactive assay has advantages in ease of application for the purpose of LVD detection and monitoring.

We have devised a novel sensitive and specific non-radioactive ILMA technique for the middle section of NT-proBNP (NT1) and the C-terminal of NT-proBNP (NT2). It was essential to separate the labelled peptides using HPLC since many peaks with the chemiluminescent label were essentially not immunoreactive. This may have been possibly due to steric hindrance from the large chemiluminescent group added to the peptides. Although both assays are sensitive and capable of detecting a few fmol of the peptides, NT1 was not a suitable assay for further development as a diagnostic tool for LVD. Moreover, 2 recent studies using a commercial radioimmunoassay kit for proBNP(22-46) (Daggubati, S. *et al.*, 1997, Cardiovascular Research, 36: 246-255;

Muders, F. *et al.*, 1997, AM. Heart J., 134: 442-449) also indicated that assay of this particular epitope was not useful in the assessment of LVD. This may have been due to inaccessibility of this particular epitope for immunodetection. In addition, recent evidence from Seidler and co-workers (Seidler, T. *et al.*, 1999, Biochem. Biophys. Res. Commun., 225(2): 495-501) indicates that this middle section of NT-proBNP has leucine zipper motifs that may lead to molecular oligomerisation, resulting in the possibility of reduced immunoreactivity or non-specific protein-protein interactions. In contrast, the assay of the C-terminal of NT-proBNP (termed NT2) revealed very significant differences in values between patients with LVD and normal controls and may be suitable for further development as a non-invasive and cost-effective diagnostic test. In addition, the ILMA described has the advantage of using non-radioactive techniques that do not require extensive laboratory radiation safety measures. The purified tracer is also stable in storage for over 6 months and is relatively inexpensive and easy to prepare. Coefficients of variation between and within assay are acceptable for a competitive assay.

In summary, we have demonstrated the possibility of using a chemiluminescent methyl acridinium ester derivative to label a small peptide and developed a competitive immunoluminometric assay for NT-proBNP which may have potential in the diagnosis and monitoring of therapy in patients with left ventricular dysfunction. More extensive studies on the usefulness of this assay are currently in progress.

EXAMPLE 2: Plasma NT-proBNP and the ECG in the assessment of ventricular systolic dysfunction in a high risk population

The main aim of this study was to test the diagnostic usefulness of circulating levels of NT-proBNP and the electrocardiogram (ECG) as indicators of left ventricular systolic

dysfunction (LVSD) in an appropriate population, ie. a cohort of patients referred by primary and secondary care physicians for echocardiography. NT-proBNP was assayed using the immunometric method of the present invention, as outlined in Example 1, *supra*.

METHODS

Subjects

We studied 249 consecutive subjects referred for echocardiography to the Cardiology Services department at Leicester Royal Infirmary, UK. Of these, 243 subjects yielded analysable echocardiograms (129 male, median age 73 years, range 20-94). Patients were selected for study if there was (i) clinical suspicion of heart failure (n=125); (ii) history of ischaemic heart disease i.e. history of myocardial infarction, presence of pathological Q wave on the ECG, physician diagnosed angina or current use of an oral or sublingual nitrate (n=85); (iii) history of hypertension (n=101); (iv) history of shortness of breath in the absence of chronic airways disease (n=132); (v) current diuretic (n=117), angiotensin converting enzyme inhibitor (n=63) or digoxin (n=29) use. Patients were excluded if there was a history of recent (within 1 month) acute myocardial infarction.

Echocardiography

Echocardiography was performed essentially as described in Example 1, *supra*. Left ventricular wall motion index (LVWMI), a regional measurement of LVSD which correlates to LVEF by radionuclide cardiography and invasive ventriculography (Berning, J. *et al.*, 1992, *supra*; Rifkin, R.D. *et al.*, 1990, Am. J. Cardiol., 65: 1485-1490) was calculated using a nine-segment model (Heger, J.J. *et al.*, 1980, *supra*). The scale used for LVWMI has been validated (Berning, J. and Stennsgaard-Hansen, F., 1990, Am. J. Cardiol., 65: 567-576; Kober, L. *et al.*, 1994, *supra*) and a linear correlation with LVEF demonstrated (Kober, L. *et al.*, 1994, Eur. Heart J. 15: 1616-1620); LVWMI multiplied by 0.3 gives an estimate of LVEF (Berning, J. *et al.*, 1992, *supra*). As in

previous large studies (Kober, L. *et al.*, 1995, *supra*) we defined LVSD as LVWMI = 1.2. LVMMI was analysed by a single investigator (ST) blind to patient details and NT-proBNP results.

Colour flow Doppler recordings on the parasternal, apical 4 chamber and the apical long axis views enabled semi-quantitative assessment of the severity of mitral, tricuspid and aortic regurgitation. Mitral regurgitation (MR) was graded as absent=0, trace = 1, moderate = 2 or severe = 3 on color flow Doppler analysis based on the area of the jet projecting into the left atrium (Miyatake, K. *et al.*, 1986, J. Am. Coll. Cardiol. 7(1): 82-88).

Immunoluminometric assay (ILMA) for NT-proBNP

A 10 ml sample of venous blood was collected into pre-chilled EDTA (1.5 mg/ml blood) tubes containing 500 IU/ml of aprotinin within 24 hours of the echocardiogram. Samples were immediately centrifuged and separated and plasma stored at -70°C until assayed using the method described in Example 1, *supra*, with antibody specific to epitope NT2 (SEQ ID NO: 1). Within and between assay coefficients of variation were 3.0 and 11.2% respectively (at 30 fmol/tube). The normal range for NT-proBNP in our laboratory is <150 fmol/ml.

Electrocardiogram (ECG)

Only ECGs performed within 2 weeks of the echocardiogram were analysed and were available for 222 / 243 (91.3%) of the subjects. Each ECG was categorised as normal, minor abnormality (sinus bradycardia, sinus tachycardia, poor R wave progression, right axis deviation, non-specific ST/T changes, 1st degree heart block, or atrial enlargement) or major abnormality (atrial fibrillation, evidence of a past myocardial infarction, voltage criteria for left ventricular hypertrophy, left axis deviation or left bundle branch block) (Davie. A.P. *et al.*, 1996, Br. Med. J., 312: 222).

Relationship between LVWMI and physiological variables

Concentrations of NT-proBNP, serum creatinine and LVWMI were not normally distributed and were log transformed before analysis. For the categorical variables gender (male/female), past history of ischaemic heart disease (IHD/no IHD) or hypertension (HT/no HT), current use of diuretic (Diuretic /no Diuretic), ACE inhibitor (ACEI/no ACEI), β-blocker (βB/ no βB) and digoxin (Dig/no Dig), ECG (normal/minor abnormality /major abnormality) and severity of mitral regurgitation (none/mild/moderate/severe), log-LVWMI were compared and 95% confidence intervals calculated for the difference in medians between groups for each variable. The strength of association between LVWMI and each of the continuous variables log NT-proBNP, age, aortic valve gradient and log creatinine concentration was quantified using the Pearson rank correlation coefficient. Predictive models for the response variable (LVWMI) were developed using multiple linear regression analysis and stepwise logistic regression analysis. All statistical analyses were carried out using the software package Minitab (Minitab Inc., PA, USA). Comparisons with $p<0.05$ were considered significant.

RESULTS

The characteristics of the study population (243 subjects) are shown in Table 2. 96 (39.5%) of the patients had LVWMI $\leq$ 1.2. Of these, 64 (66.6%) were taking a diuretic, 43 (44.7%) an ACE inhibitor, 76 (79.1%) gave a history of shortness of breath and 71 (73.9%) had clinical evidence of heart failure. Concentration of NT-proBNP (median [range]) was higher in subjects with LVWMI $\leq$ 1.2 (509.6 fmol/ml [195.2-1619.3]) compared to those with of LVWMI $\geq$ 1.3 (255.2 fmol/ml [65.5- 1175.9] fmol/ml; $p<0.0001$).

Table 2. Characteristics of the study population.	
Total patients	243
Age, median [range]	73 years[20-94]
Male	129
History of shortness of breath	132
History of Ischaemic heart disease	85
History of Hypertension	101
Clinical heart failure	102
Diuretic use	117
ACE inhibitor use	63
Digoxin use	29
Beta-blocker use	32
Creatinine, median[range]	106 μmol/L [37-316]
Wall Motion Index, median [range]	1.6 [0.2-2]

Determinants of LVWMI - Univariate analysis

On univariate analysis for the whole study population there was a negative correlation between log NT-proBNP and LVWMI ($r=-0.624$, $p<0.001$) (Figure 4). LVWMI also correlated with age ($r= -0.15$, $p=0.01$), serum creatinine ($r=-0.29$, $p<0.001$), past history of IHD ($r=0.29$, $p<0.001$), male gender ($r=0.23$, $p<0.001$) and current treatment with diuretic ($r=0.34$, $p<0.001$), or ACE inhibitor ($r=0.38$, $p<0.001$).

132 (54%) patients were treated with ACEI and/or diuretic (48 both, 69 diuretic alone, 15 ACEI alone). Those treated were older (74±10 yrs) than those untreated (66±17 yrs, $p< 0.0005$). NT-proBNP levels (fmol/ml; Median, Range) were similar in untreated (368, 78-1047) compared to patients receiving either ACEI or diuretic (355, 65-1619), ACEI alone (382, 84-1619), diuretic alone (398, 65-1609) or both (332, 124-1293)($p=0.981$,

Kruskal-Wallis). LVWMI did not differ among groups (p=0.941, Kruskal-Wallis). The correlation between log NT-proBNP and LVWMI was similar for both treated (r=-0.661,p< 0.005) and untreated (r=-0.584, p<0.005) patients (Figure 4).

Multivariate analysis

On multiple regression analysis in the whole population, log NT-proBNP (p<0.001), age (p=0.015), diuretic use (p=0.002), ACE inhibitor use (p=0.001) and male gender (p=0.026) were independently associated with low LVWMI. Serum creatinine (p=0.09) and abnormal ECG (p=0.27) were not predictors of LVWMI in the multivariate model. Using best subsets analysis, log NT-proBNP alone (R^2=39%) was a better predictor of LVWMI than any other single factor (ACE Inhibitor R^2= 15%; mitral regurgitation R^2 = 15%; diuretic R^2= 12%) factor. The predictive valuc of the model was improved slightly by consideration of combinations of variables, all of which included log NT-proBNP: log NT-proBNP + ACE inhibitor R^2=44%; log NT-proBNP + ACE inhibitor + male gender R^2=47%; log NT-proBNP + ACE inhibitor + male gender + diuretic R^2=48%.

Independent predictors of LVWMI in patients treated with ACEI and/or diuretic were NT-proBNP (p<0.001) and age (p<0.001). Using best subsets analysis, log NT-proBNP alone (R^2=26.3%) was again the best predictor of LVWMI than any other single or combination of factors. In untreated patients only NT-proBNP (p< 0.001) independently predicted WMI. On best subsets analysis, log NT-proBNP alone (R^2=42.8%) was the best predictor of LVWMI. Thus, in both treated and untreated patients, log NT-proBNP accounted for a substantial proportion of the total variance in LVWMI. There was a weak and statistically non-significant correlation between plasma NT-proBNPand serum creatinine concentration (r=0.156, p=0.08).

The ECG in screening for LVSD

In this largely hospital based population, a normal ECG was found in 36/222 (16%), a minor abnormality in 36/222 (16%) and a major abnormality in 150/222 (68%). Of the 36 patients with normal ECG, 18 had LVWMI = 2. Six (17%) had LVWMI of 1.3-1.9 and 12 (33%) LVWMI of ≤ 1.2. Thus the ECG alone would have failed to identify 18 patients in whom LV function was impaired. In all of these patients NT-proBNP was > 275 fmol/ml. Of the further 36 patients with minor ECG abnormalities, 10 (28%) had LVWMI of 1.3-1.9. NT-proBNP was > 275 fmol/ml in 7/10. 15/36 (42%) of patients had LVWMI of ≤ 1.2 and of these NT-proBNP was > 275 fmol/ml in 13. Of the 150 patients with major ECG abnormalities 52 (35%) had LVWMI = 2, in 23 (44%) of whom NT-proBNP was > 275 fmol/ml. 36/150 (24%) had LVWMI of 1.3-1.9, in 26 (72%) of whom NT-proBNP was > 275 fmol/ml. Of the 150 patients whose ECG showed a major abnormality 52 had LVWMI = 2, in 29 of whom NT-proBNP was < 275 fmol/ml. NT-proBNP was > 275 fmol/ml in all 62 patients with major ECG abnormality and LVWMI of ≤ 1.2.

Influence of mitral regurgitation

MR was graded as 0 in 44 (18%), 1 in 145 (60%), 2 in 40 (16%) and 3 in 14 (6%) patients. The estimated severity of MR correlated with plasma NT-proBNP ($r=0.389$, $p<0.0005$). Mean NT-proBNP differed among groups ($p<0.0005$, Kruskal-Wallis) and between all pairs except MR=2 and MR=3. (Figure 5). However there was a correlation between the severity of MR and WMI ($r=-0.365$, $p<0.0005$). Thus although elevated NT proBNP largely reflects reduced WMI it is affected by the presence and severity of MR.

DISCUSSION

Our study has confirms the relationship between plasma levels of natriuretic peptides and measures of left ventricular. Moreover our study indicates that plasma NT-proBNPremains a strong predictor of left ventricular function when confounding variables such as renal function and concomitant drug therapy are taken into account.

Our study also indicates that, at least in a population at relatively high risk of left ventricular dysfunction, consideration of the ECG does not add to the predictive value of plasma NT-proBNPlevel.

Our study has confirmed a linear relationship between NT-proBNP and left ventricular function as assessed by LVWMI, a relationship previously reported for LV ejection fraction (Richards, M.A. *et al.*, 1998, *supra*). We have demonstrated the strong, independent predictive value of plasma NT-proBNP in the identification of LV dysfunction. Previous studies on the use of natriuretic peptides for detecting LVSD have utilized echocardiographic ejection fraction and fractional shortening (Cowie. M.R. *et al.*, 1997, Lancet, 350: 1349-1353; Richards, M.A. *et al.*, 1998, *supra*; Choy, A.M.J. *et al.*, 1994, Br. Heart J., 72: 16-22); or radionuclide ventriculography (Richards, M.A. *et al.*, 1998, *supra*). Echocardiographic measures of LV dysfunction have with rare exceptions failed to predict prognosis in multivariate models (Cowburn, P.J. *et al.*, 1998, Eur. Heart J., 19: 696-710). This perhaps reflects the inaccuracy of current methods for measuring ejection fraction. On the other hand, the LVWMI is a relatively simple, robust and reproducible measurement (Kober, L. *et al.*, 1994, *supra*; Kober *et al.*, 1995, *supra*) and the nine segment model has been shown to contain prognostic information in patients with HF (Madsen, B.K. *et al.*, 1996, Cardiology, 87: 250-256).

Concerns have been expressed that drug treatment, in particular diuretic or ACE inhibitor therapy, β-blockers and digoxin may modify plasma levels of natriuretic peptides and nullify their potential as markers for LVSD. We have observed the correlation of NT-proBNP with LVWMI to be as strong in the group who were being treated with a diuretic or an ACEI when compared to those patients who were receiving neither. Similarly we detected no influence of concomitant β-blocker or digoxin therapy. Although our study does not indicate whether NT-proBNP levels had been altered by these therapies, our

data suggests that the utility of this measurement is not significantly reduced in the presence of appropriate treatment for LVSD or ischaemic heart disease.

NT-proBNP had a sensitivity of approximately 95% and specificity of 55% for the detection of LVSD. The sensitivity and specificity respectively of various natriuretic peptides has been reported as 77% and 87% (McDonagh, T.A. *et al.*, 1998, Lancet, 351: 9-13), 97% and 84% (Cowie, M.R. *et al.*, 1997, *supra*) for BNP in primary care, 91% and 72% for N-terminal BNP following MI (Richards, M.A. *et al.*, 1998, *supra*). The variability in reported sensitivity and specificity is likely to reflect not only differences among assay procedures but also differences in the selection of study populations. Of more clinical importance is the high negative predictive value (93%) of NT-proBNP in the diagnosis of LVSD in our study.

It has thus been demonstrated in this study in a high risk population that NT-proBNP is a powerful predictor of the degree of LVSD as measured by LVWMI. NT-proBNP strongly predicts LVWMI even in the presence of concomitant drug therapy. Moreover we have shown that consideration of the ECG alone in such patients may be misleading and that consideration of the ECG in addition to plasma NT-proBNPlevel adds little to the identification of patients with LVSD.

EXAMPLE 3: Profile of plasma NT-proBNP following acute myocardial infarction and correlation with left ventricular systolic dysfunction

The aims of this study were to describe the temporal pattern of plasma N-terminal pro-brain natriuretic peptide using the method according to the present invention, to examine the optimum time of sampling and to compare plasma N-terminal pro-brain natriuretic

peptide NT2 (SEQ ID NO: 1) to clinical criteria in terms of identification of impaired left ventricular systolic function following acute myocardial infarction.

METHODS

Subjects

We studied 60 patients admitted to the Coronary Care Unit of Leicester Royal Infirmary, UK, with a diagnosis of Q-wave AMI. Serial blood samples were taken from each subject in each of the following periods: 14-48 hours, 49-72 hours, 73-120 hours, 121-192 hours following AMI and at a clinic visit in survivors.

Echocardiography

Echocardiographic assessment of wall motion index (WMI), a measure of left ventricular systolic dysfunction, was made in 58 of the 60 patients during admission (WMI-1;Median day 4.5, range 2-6) and at the clinic visit (WMI-2; Median day 50, range 20-73) in 52/56 survivors. Echocardiography was performed as described in Examples 1 and 2, *supra*.

Blood sampling

20 ml of venous blood was taken at each of 5 different time periods following the index AMI. Blood was transferred into pre-chilled EDTA (1.5 mg/ml blood) tubes containing 500 IU/ml of aprotinin. Samples were immediately centrifuged at 4°C and plasma separated and then stored at -70°C until assayed.

Assay for N-BNP

The methodology for assaying NT-proBNP using epitope NT2 (SEQ ID NO: 1) according to the present invention is as described in Example 1, *supra*.

Statistical analysis

Assessment was made of the strength of relationship between left ventricular systolic function (WMI) during and after hospitalization and N-BNP measured at each time interval. The relationship with WMI of a number of additional clinical and laboratory variables was investigated. Predictive models for the response variable (WMI) were developed using multiple linear regression analysis and stepwise logistic regression analysis.

Concentrations of N-BNP, age, plasma creatinine, plasma glucose, peak creatine kinase, and WMI scores were not normally distributed and were log transformed before analysis. Comparisons of N-BNP levels at different time points was by analysis of variance (ANOVA) with correction for multiple measures. Other comparisons were by Students t-test. Comparisons with $p<0.05$ were considered significant. All statistical analyses were carried out using the software package Minitab (Minitab Inc., PA, USA). All results are expressed as means $\pm$ 1 SD. N-BNP levels are expressed in fmol/ml.

RESULTS

We studied 60 patients (45 Male, median age 63.5 years, range 36-87, 39 anterior AMI). Biochemical and echocardiographic data are in Table 3. A past history of myocardial infarction, angina, hypertension and diabetes mellitus was obtained in 4 (6.7%), 10 (16.7%), 16 (26.6%), and 4 (6.7%) respectively. None had a previous history of heart failure. Twenty four (40%) and 35 (58%) patients had radiological and/or clinical evidence of heart failure during hospitalisation. In terms of treatment, 52 (86.7%) received thrombolysis, 33 (55%) diuretic, 38 (63.3%) angiotensin converting enzyme (ACE) inhibitor, 33 (55%) beta blocker and 2 (3.3%) digoxin. By 6 weeks 4 (6.7%) had died. At this point 35/54 (64.8%) had WMI $\leq$ 1.2. A 6 week echo was not obtained in 2/56 survivors.

Table 1: Biochemical and echocardiographic indices of study population

Biochemistry	**Mean (range)**
Plasma Sodium (mmol/l)	137.2 (127-146)
Plasma Urea (mmol/l)	6.3 (2.9-11)
Plasma Creatinine (μmol/l)	104.7 (46-177)
Plasma Glucose (mmol/l)	9.3 (5.7-30.9)
Peak CK (iU/L; NR < 200)	2153 (487-8922)
Echocardiographic indices	
WMI-1	1.2 (0.2-2)
WMI-2	1.5 (0.5-2)

(NR = Normal Range)

Profile of plasma N-BNP

Plasma N-BNP levels (fmol/ml), measured according to the method of the present invention, were elevated at 14-48h (748 $\pm$ 170), 49-72h (579 $\pm$ 138), 73-120h (450 $\pm$ 124), 121-192 h (823 $\pm$ 257) and at the clinic visit (807 $\pm$ 176). Plasma N-BNP fell between 14-48 hours and 73-120 hours ($p=0.007$) and rose between 73-120 hours and the clinic visit ($p=0.01$, ANOVA). This biphasic response was seen only following anterior AMI. N-BNP levels did not differ among time points following inferior AMI in whom plasma levels were similar at all times ($p=0.6$, ANOVA) (Figure 6).

At all time points N-BNP levels (fmol/ml) were higher in anterior compared to inferior MI (1000 $\pm$ 255 vs 292.8 $\pm$ 41.1, $p<0.005$ at 14-48 h; 732 $\pm$ 199 vs. 261.4 $\pm$ 44.6, $p<0.05$ at 49-72 h; 595 $\pm$ 196 vs. 214.1 $\pm$ 29.5, $p<0.005$ at 73-120 h; 1140 $\pm$ 374 vs. 187.4 $\pm$ 22.3, $p<0.005$ at 121-192 h and 1162 $\pm$ 268 vs. 246.4 $\pm$ 40.9, $p<0.005$ at the clinic visit).

N-BNP levels did not differ between those patients with clinical or radiological evidence of heart failure and those without (785 ± 383 vs 485 ± 238, p=0.15 at 14-48 h; 693 ± 467 vs. 505 ± 761, p=0.55 at 49-72 h; 686 ± 758 vs.317 ± 314, p=0.28 at 73-120 h; 1091 ± 794 vs. 674 ± 1698, p=0.45 at 121-192 h and 597 ± 836 vs. 908 ±385, p=0.33 at the clinic visit.

N-BNP and Wall Motion Index score

There was a strong correlation between WMI-1 and WMI-2 (r= 0.756, p<0.0001). WMI-2 was significantly higher than WMI-1 (P<0.0001, Table 3). N-BNP at 14-48 hours correlated with N-BNP at 49-72 hours (r= 0.57, p<0.0001), 73-120 hours (r= 0.68, p<0.0001), 121-192 hours (r= 0.56, p<0.0001) and at the out patient visit (r = 0.31, p<0.05). There was a consistent correlation between WMI-1 and N-BNP: 14-48h (r = - 0.52, p<0.005), 49-72h (r = - 0.57, p<0.005), 73-120h (r = - 0.64, p<0.005) (Figure 7) and 121-192 h (r = - 0.57, p<0.005). The correlation of WMI-2 with N-BNP was less strong and only significant for N-BNP measured at 14-48h (r= - 0.29, p<0.05) and at 73-120h (r= - 0.41, p=0.005). There was no correlation between N-BNP measured at the clinic visit and WMI-2 (r=-0.098, p=0.526). There was no significant correlation between N-BNP levels at any time and either peak creatine kinase or plasma creatinine.

Influence of treatment

Both WMI-1 (1.1 ± 0.3 vs 1.6 ± 0.4, p=0.0002) and WMI-2 (1.4 ± 0.4 vs 1.8 ± 0.3, p=0.0002) were lower in patients who received ACE inhibitor therapy. N-BNP was higher in patients who received ACE inhibitor therapy compared to those who did not (997 ± 752 vs 280 ± 195 at 14-48 hr; 626 ± 761 vs 194 ±75 at 49-72 hr; 403 ± 304 vs 170 ± 82 at 73-120 h, 962 ± 875 vs 214 ± 123 at 121-192 h; 1158 ± 1078 vs 353 ± 450 at the clinic visit; all p < 0.005).

Predictors of Wall Motion Index

1. WMI-1

We considered N-BNP at 14-48h, 49-72h, 73-120 h, 121-192h, age, gender, past history of AMI, history of hypertension, history of diabetes mellitus, ECG site of infarct, plasma creatinine, peak creatine kinase, clinical heart failure and radiological heart failure in multivariate models for the predictors of WMI-1. On best subsets analysis the strongest independent predictor of WMI-1 was N-BNP at 73-120h (R^2=39%, $p<0.005$). The addition of anterior site of infarct improved diagnostic accuracy (R^2=49%, $p<0.005$) of the model. Other significant predictors of WMI-1 were history of diabetes mellitus ($p<0.05$) and history of previous AMI ($p<0.05$), which when combined with N-BNP at 73-120h and anterior site of infarct increased the accountable variance in WMI-1 (R^2=58%, $p<0.005$). When only anterior site, N-BNP at 73-120h, history of MI and history of diabetes were included in the analysis, both anterior site of infarction ($p=0.002$) and N-BNP at 73-120h ($p=0.005$) remained independent predictors of WMI-1. In those patients without clinical or radiological evidence of heart failure, the correlation of N-BNP at 72-120 hours with WMI-1 remained strong ($r=-0.494$, $p=0.005$).

In addition to comparing the relative predictive value of N-BNP measured at all time points, we analysed the predictive value of N-BNP at each individual time. When added as before to the variables age, gender, past history of AMI, history of hypertension, history of diabetes mellitus, ECG site of infarct, plasma creatinine, peak creatine kinase, clinical heart failure and radiological heart failure, N-BNP was superior to all other variables at 49-72 hrs (R^2=32.5%), 73-120 hrs (R^2=40.5%) and 121-192 hrs (R^2=32.9%)(All $p<0.005$). At 14-48 hrs the predictive value of N-BNP was lower than at other times (R^2=27.4%) and less than that of anterior site of infarction (R^2=32.2%). The combination of anterior site of infarction and N-BNP at 14-48 hrs increased the predictive value of the model (R^2=42.4%).

2. WMI-2

We considered the same variables in a model for the predictors of WMI-2. The strongest independent predictor of WMI-2 was again N-BNP at 73-120h (R^2=15%). The addition of anterior site of infarct (R^2=20%, $p<0.05$) and past history of AMI (R^2=26%, $p<0.005$) improved diagnostic accuracy. When analysis was restricted to the variables anterior site, N-BNP at 73-120h, history of MI and history of diabetes, anterior site of infarction ($p=0.008$) displaced N-BNP at 73-120h ($p=0.03$) as the strongest predictor of WMI-2 although both retained independent value.

Predictors of outcome

N-BNP level at 73-120h (R^2=17.7%, $p=0.005$) and history of previous myocardial infarction (R^2=5.3%, $p<0.05$) were the only independent predictors of poor outcome (WMI-2 $\leq$ 1.2 or death by 6 weeks), the combination accounting for 25% of the variation in response. The only independent predictors of death alone were plasma N-BNP at 73-120 h (R^2=35%, $p=0.005$) and at 121-192 h (R^2=14%, $p=0.05$). The addition of anterior site of infarction to plasma N-BNP at 73-120 h improved the accuracy of the model in predicting death (R^2=40%, $p<0.05$).

Patients with poor outcome (LVSD or death) had higher N-BNP levels compared to the rest at all times (Figure 8). N-BNP > 240 fmol/ml at 73-120h had a positive predictive value of 74% and a negative predictive value of 61% in predicting WMI-1 $\leq$ 1.2, and positive predictive value of 41% and negative predictive value of 91% in predicting death or WMI-2 $\leq$ 1.2 (Table 4). The corresponding values using N-BNP at 14-48h were 78% and 58% for WMI-1 $\leq$ 1.2 and 37% and 75% for death or WMI-2 $\leq$ 1.2.

Table 4: Sensitivity, specificity, positive predictive value and negative predictive values of clinical heart failure, radiological heart failure and plasma NT-proBNP at 73-120 hours following myocardial infarction for the prediction of death or WMI-2 ≤ 1.2.

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Clinical heart failure	63	70	45	82
Radiological heart failure	63	48	48	76
Both clinical and radiological heart failure	75	42	34	81
N-BNP > 240 (fmol/ml)	85	56	41	91

N-BNP > 500 fmol/ml at any time point during the hospital stay had a positive predictive value of 47% and a negative predictive value of 100% in predicting death or WMI-2 of ≤ 1.2.

DISCUSSION

This study reports for the first time the optimum time for measurement of N-BNP following AMI. Our results indicate that N-BNP measured later in hospitalisation is superior to measurement at earlier times as regards identifying patients with significant left ventricular systolic dysfunction soon after AMI and with poor echocardiographic or clinical outcome in the 6 weeks following AMI. N-BNP measured during the first 2 days after AMI had similar predictive values for WMI-1 but was less useful than the later measurement in predicting death or left ventricular systolic dysfunction in the weeks after discharge. N-BNP at 73-120h was superior to clinical, radiological or electrocardiographic parameters in the identification of impaired left ventricular systolic

function.

Profile of plasma N-BNP

Our study confirms that plasma levels of N-BNP are elevated in the early stages following AMI, peaking within 48 hours before declining over the next 48 hours. This is followed, after anterior infarction, by a secondary rise in plasma N-BNP at around day 5, maintained 6 weeks later. The biphasic pattern of plasma N-BNP is, not surprisingly, analogous to the pattern of secretion observed for BNP-32 following AMI (Morita, E. *et al.*, 1993, Circulation, 88: 82-91). This supports the assertion that the initial rise corresponds to release of stored of N-BNP following tissue necrosis in the early phase of AMI with a secondary rise paralleling the ensuing development of infarct expansion and evolution of LVSD. The strongest correlation with impairment of left ventricular function was with N-BNP at 72-120 hours, ie at the trough of the biphasic response. While this may appear surprising, we suggest that this time point identifies those in whom N-BNP shows the least fall from the initial peak. This may indicate either large amounts of myocardial necrosis, early initiation of the process of ventricular remodelling, or both. Thus not only the absolute magnitude but also the pattern of N-BNP response is related to the size of infarct and development of LVSD subsequent to AMI.

Both BNP (Richards, A.M. *et al.*, 1999, Heart, 81: 114-120) and N-BNP (Richards, A.M. *et al.*, 1998, *supra*) predict left ventricular ejection fraction during and some months following AMI, each of these studies assessing the value of a single peptide level measured in the early post infarct period. Our study suggests that N-BNP measured later rather than earlier in the acute phase is a stronger predictor of poor clinical outcome as measured by death or LV systolic dysfunction. This finding has implications for the potential use of N-BNP as a marker for LV dysfunction following AMI.

N-BNP and assessment of LV function

We found a strong correlation between levels of N-BNP and WMI during hospitalization, observed despite wide variation in clinical characteristics and patterns of acute management of patients. Our data has also demonstrated a correlation between of N-BNP measured 4-5 days after AMI and WMI 6 weeks later, in agreement with previous studies (Richards, A.M. *et al.*, 1998, *supra*). Although the correlation was statistically significant, the absolute strength of association was only moderate ($r = -0.41$, $p=0.005$).

The predictive value of a test in confirming or excluding the presence of a condition is of more clinical relevance. In this context, our finding that N-BNP better identifies LV systolic dysfunction than does radiological or clinical evidence of heart failure or the combination of these is an important finding of this study.

The clinical usefulness of a test depends not only on the strength of association between the marker being assayed and the condition being sought but also on the robustness of the test in comparison to currently applied criteria. Our data indicate that for the prediction of death or WMI-2 $\leq$ 1.2 measurement of N-BNP better identifies patients at high risk (and who might benefit from appropriate early pharmacological therapy) than do clinical or radiological assessment. The strength of N-BNP is emphasized by our demonstration of its superiority to anterior site of infarction in multivariate models of factors determining WMI. Our data confirms the high negative predictive value of N-BNP allowing identification of patients at low risk. However we do not suggest that patients with clinical evidence of heart failure but "normal" N-BNP following AMI should be denied appropriate, ie ACE inhibitor, therapy.

Assay of N-BNP

The choice of antibody and the method of peptide measurement may have important implications for the development of a clinically useful assay. Levels of N-BNP recorded

in our study, utilizing an antibody against the C-terminal of N-BNP (the NT2 epitope of the present invention) are considerably higher than the values determined in a previous study using an antibody directed against the N terminal of N-BNP (Richards, A.M. *et al.*, 1998, *supra*). The N-terminal domains of preproBNP have been demonstrated to oligomerise through leucine 'zipper-like coiled-coil' motifs (Seidler, T. *et al.*, 1999, *supra*), a short bundle of peptide α-helices wound into a superhelix. It is possible that an antibody directed against N-terminal domains of N-BNP is potentially hindered from binding to its equivalent amino acid sequence. C-terminal domains may be more readily accessible and detectable by immunoassay. Such differences in the immunoreactivities of the N- and C-terminals of N-BNP are likely to account for the disparity between the concentrations of N-BNP observed in previous work (Richards, A.M. *et al.*, 1998, *supra*) and the current study. Furthermore, other studies that have employed antibodies towards the mid-section of N-BNP have failed to demonstrate any diagnostic value for detection of LVSD (Daggubati S. *et al.*, 1997, Cardiovasc. Res., 36: 246-255; Muders F. *et al.*, 1997, Am. Heart J., 134: 442-449; Yandle T. *et al.*, 1999, Lancet, 353: 1068-1069), further emphasizing the diagnostic utility of the present invention.

CLAIMS

1. A peptide identical to an epitope having the amino acide sequence of SEQ ID NO: 1.

2. A peptide according to claim 1 having the amino acide sequence of SEQ ID NO: 1.

3. A peptide according to either one of the preceding claims for use in a method of diagnosis of NT-proBNP.

4. The use of a peptide according to either one of claims 1 or 2 in the manufacture of a diagnostic test for NT-proBNP.

5. A method of manufacture of a diagnostic test for NT-proBNP, characterised in the use of a peptide according to either one of claims 1 or 2.

6. The use of a peptide according to either one of claims 4 or 5, the diagnostic test being for left ventricular dysfunction.

7. The use of a peptide according to claim 6, the diagnostic test being performed within the range of about 70 to 120 hours following acute myocardial infarction.

8. A diagnostic test method for left ventricular dysfunction, comprising the steps of:

i) determining the quantity of a peptide according to either one of claims 1 or 2 in a sample from a patient;

ii) comparing the level of peptide determined in step (i) with the level of peptide determined in another patient having a known left ventricular function; and

iii) correlating the results of comparison step (ii) with left ventricular dysfunction of the patient.

9. Antibody specific against a peptide according to either one of claims 1 or 2.

10. The use of antibody according to claim 9 in the manufacture of a diagnostic test for NT-proBNP.

11. A method of manufacture of a diagnostic test for NT-proBNP characterised in the use of antibody according to claim 9.

12. The use of antibody according to either one of claims 10 or 11, the diagnostic test being for left ventricular dysfunction.

13. The use of antibody according to claim 12, the diagnostic test being performed within the range of about 70 to 120 hours following acute myocardial infarction.

14. A diagnostic test method for NT-proBNP comprising the steps of:

i) reacting antibody specific to a peptide according to either one of claims 1 or 2 with a sample from a patient;

ii) detecting an antibody-antigen binding reaction; and

iii) correlating detection of the antibody-antigen binding reaction with the presence of NT-proBNP.

15. The use of antibody specific against a peptide according to either one of claims 1 or 2 together with antibody specific against a different epitope displayed by NT-proBNP in the manufacture of a two-step diagnostic test for NT-proBNP.

16. A diagnostic test method for NT-proBNP comprising the steps of:

i) reacting with a sample from a patient a first antibody specific to either one of the group of a peptide according to either one of claims 1 or 2 and a different epitope displayed by NT-proBNP;

ii) reacting with sample bound to antibody in step (i) a second antibody specific to the other of the group of a peptide according to either one of claims 1 or 2 and a different epitope displayed by NT-proBNP;

iii) detecting binding of the antibody of step (ii) with antigen; and

iv) correlating the results of detection step (iii) with the presence of NT-proBNP.

17. A diagnostic test method according to claim 16, the second antibody being labelled with a chemiluminescent marker.

18. The use of antibody or a diagnostic test method according to either one of claims 15 or 16, the antibody not specific against a peptide according to either one of claims 1 or 2 being specific against a peptide identical to an epitope having the sequence of SEQ ID NO: 2.

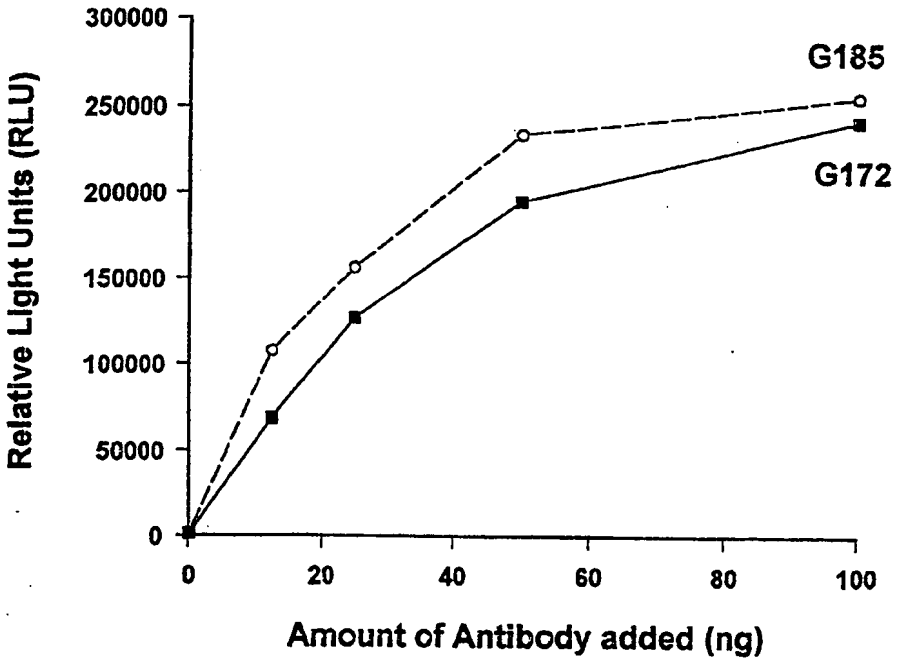

300000
250000
200000
150000
100000
50000
0
Relative Light Units (RLU)
0
20
40
60
80
100
Amount of Antibody added (ng)
G185
G172

Figure 1

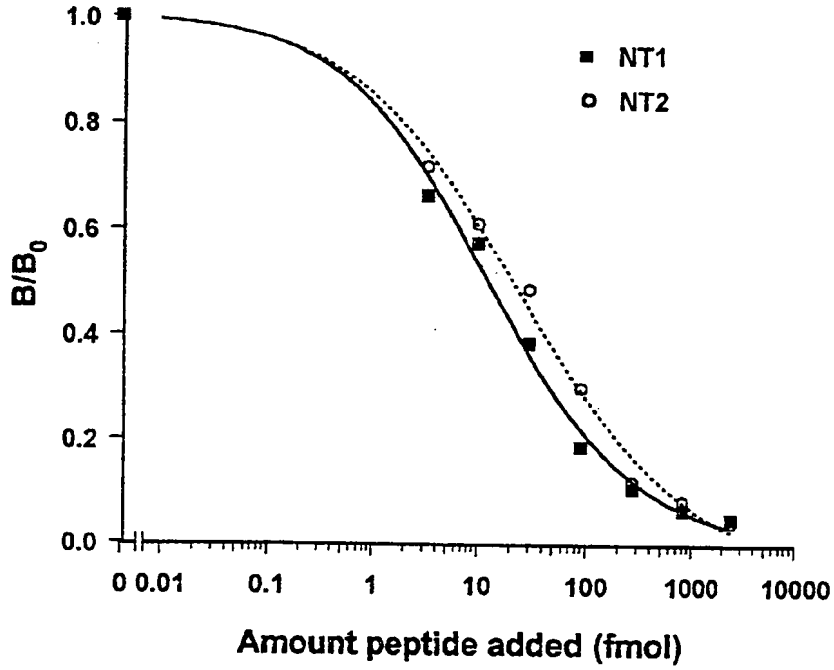

1.0
0.8
0.6
0.4
0.2
0.0
B/B0
NT1
NT2
0 0.01
0.1
1
10
100
1000
10000
Amount peptide added (fmol)

Figure 2

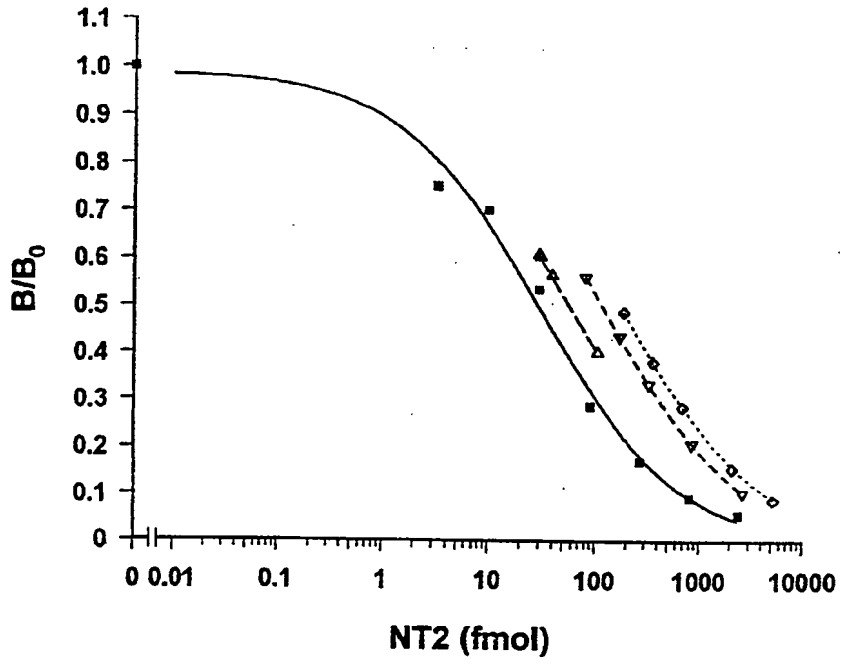

1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
B/B0
0 0.01 0.1 1 10 100 1000 10000
NT2 (fmol)

Figure 3

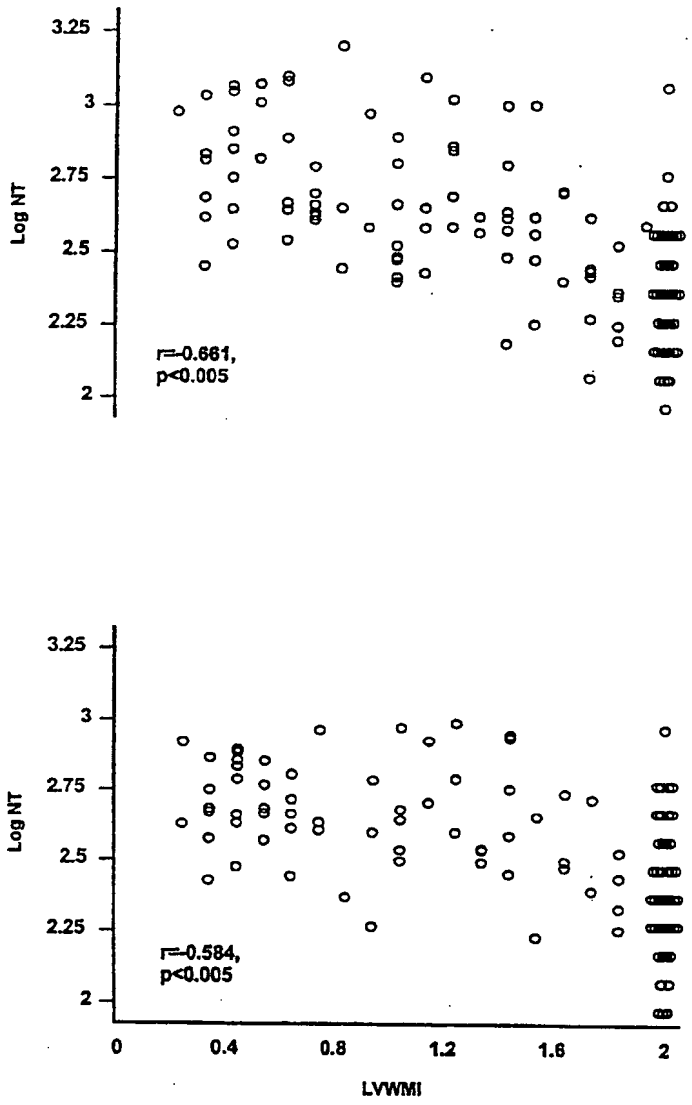

3.25
3
2.75
2.5
2.25
2
Log NT
r=-0.661,
p<0.005
3.25
3
2.75
2.5
2.25
2
Log NT
r=-0.584,
p<0.005
0
0.4
0.8
1.2
1.6
2
LVWMI

Figure 4

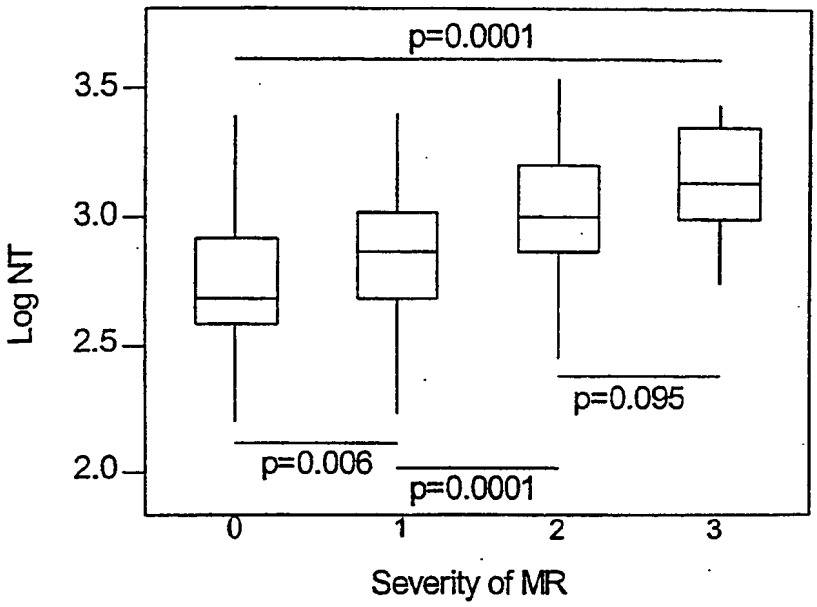

p=0.0001
3.5
3.0
2.5
2.0
Log NT
p=0.095
p=0.006
p=0.0001
0
1
2
3
Severity of MR

Figure 5

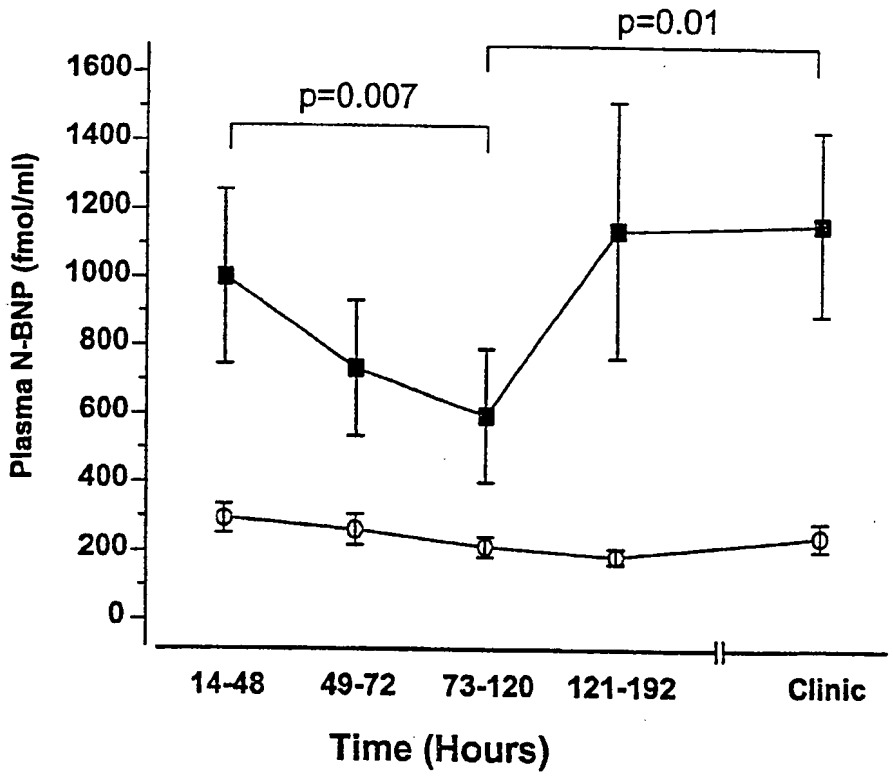

p=0.01
p=0.007
1600
1400
1200
1000
800
600
400
200
0
Plasma N-BNP (fmol/ml)
14-48
49-72
73-120
121-192
Clinic
Time (Hours)

Figure 6

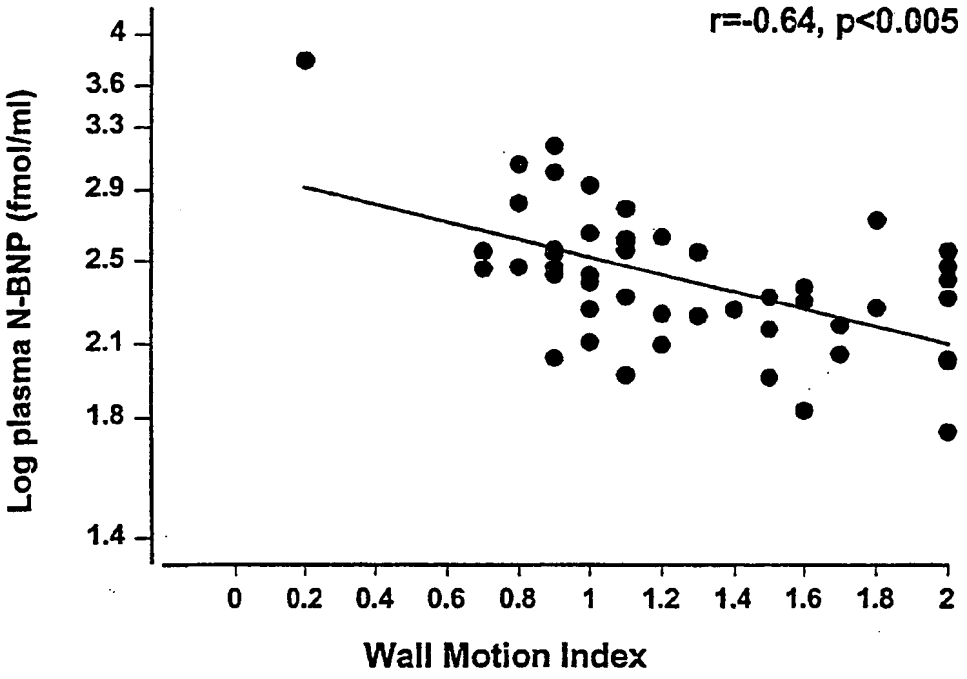
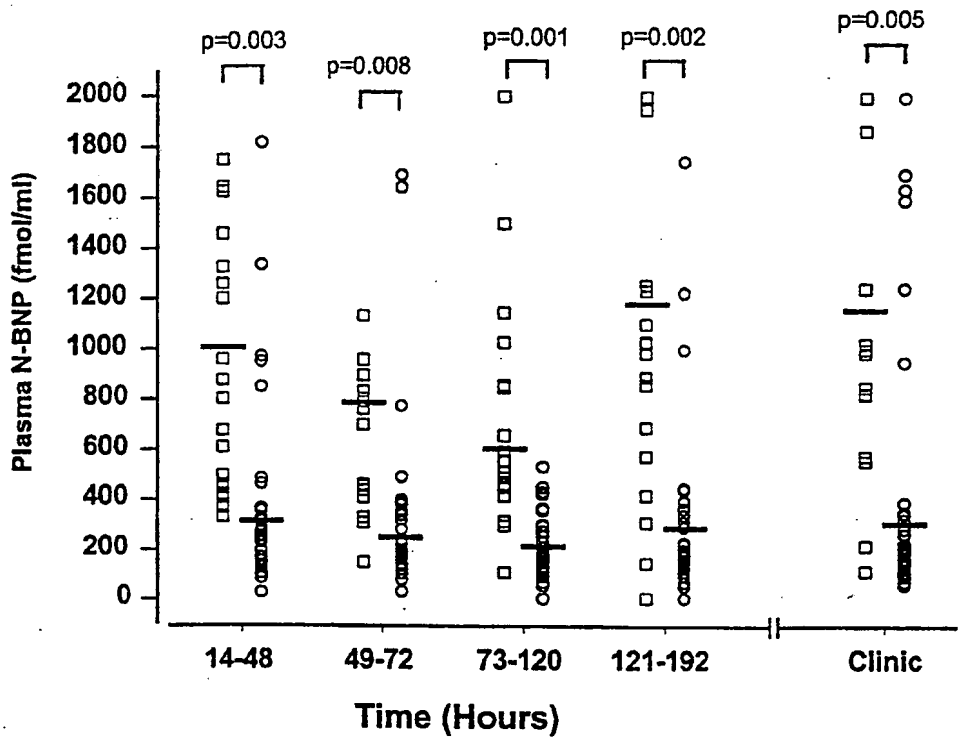

r=-0.64, p<0.005
Log plasma N-BNP (fmol/ml)
4
3.6
3.3
2.9
2.5
2.1
1.8
1.4
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
Wall Motion Index

Figure 7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p=0.008
p=0.001
p=0.002
p=0.005
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Plasma N-BNP (fmol/ml)
14-48
49-72
73-120
121-192
Clinic
Time (Hours)

Figure 8

SEQUENCE LISTING

<110> The University of Leicester

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 99/04160

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/58 G01N33/68 C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 786 163 A (HALL CHRISTIAN) 28 July 1998 (1998-07-28) column 3, line 24 - line 49; claims; examples 1,2	1,3-6, 9-12
A	WO 97 32900 A (SCARDINA JAN MARIAN ;SCIOS INC (US); LIM GARRET A (US); MISCHAK RO) 12 September 1997 (1997-09-12) column 2, line 6 - line 18; claims; examples	1-18

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 2000	12/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

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INTERNATIONAL SEARCH REPORT

Intex onal Application No
PCT/GB 99/04160

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	A. CLERICO ET AL.: "Circulating levels of cardiac natriuretic peptides (ANP and BNP) measured by highly sensitive and specific immunoradiometric assays in normal subjects and in patients with different degrees of heart failure" JOURNAL OF ENDOCRINOLOGICAL INVESTIGATION, vol. 21, March 1998 (1998-03), pages 170-179, XP000891965 page 175, right-hand column, paragraph 3 -page 177, right-hand column, paragraph 1	1,3-6, 9-12
P,X	D. HUGHES ET AL.: "An immunoluminometric asay for N-terminal pro-brain natriuretic peptide: develop,ment of a test for left venticular dysfunction" CLINICAL SCIENCE, vol. 96, no. 4, April 1999 (1999-04), pages 373-380, XP002136252 the whole document	1-18

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 99/04160

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		CA 2136961 A	09-12-1993
		DE 69321955 D	10-12-1998
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		WO 9324531 A	09-12-1993
		JP 7507210 T	10-08-1995
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		CA 2248140 A	12-09-1997
		EP 0914344 A	12-05-1999